Stem Cell Reports, Volume *2*

Supplemental Information

Simvastatin Promotes Adult Hippocampal Neurogenesis by Enhancing Wnt/β-Catenin Signaling

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INVENTORY OF SUPPLEMENTAL INFORMATION

1. Figure S1, related to Figure 2.

   XAV939 blocks simva mediated enhancement of WNT signaling in aNPCs.

2. Figure S2, related to Figure 3.

   Simva does not affect cCASP3-associated apoptosis.

3. Figure S3, related to Figure 4.

   Simva enhances WNT signaling in the SH-SY5Y cell line, as shown previously in other cell lines.

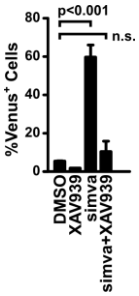
4. Figure S4, related to Figure 4.

   The siRNAs used for *HMGCR* knockdown are effective at reducing the number of transcripts of this gene.

5. Supplemental Experimental Procedures

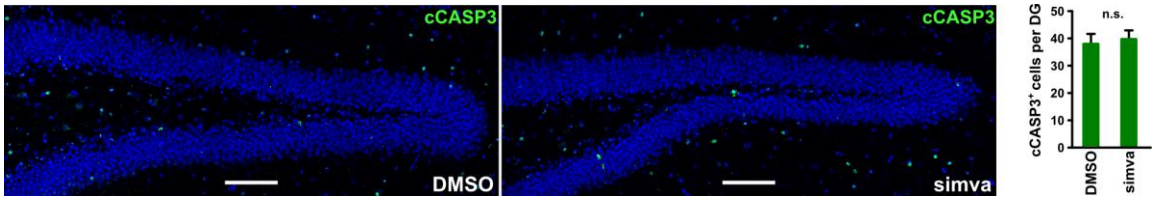
SUPPLEMENTAL FIGURES

Figure S1, related to Figure 2.



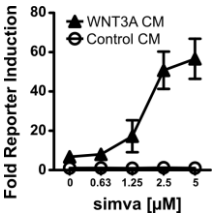
XAV939 blocks simva mediated enhancement of WNT signaling in aNPCs. Quantification of Venus^{+} cells in aNPCs treated with DMSO, 5 μM simva, 2.5 μM XAV939, or simva and XAV939, all in the presence of rcWNT3A (20 ng/mL) Results are presented as Mean ± SEM, n = 5 independent wells.

Figure S2, related to Figure 3.



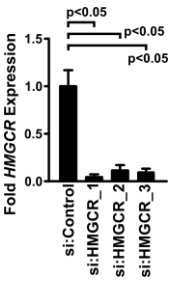
Simva does not affect cCASP3-associated apoptosis. Staining for apoptotic marker cCASP3 in the DG of mice treated with simva or DMSO, counterstained with DAPI (Scale bars, 100 μm), and quantification of cleaved CASP3 cell number. Results are presented as Mean ± SEM, n = 8 mice per group. Statistical analysis performed with Student's unpaired t-test.

Figure S3, related to Figure 4.



Simva enhances WNT signaling in the SH-SY5Y cell line, as shown previously in other cell lines. BAR luciferase reporter activity in SH-SY5Y cells treated with a dose series of simva, in combination with either WNT3A CM or control CM. Results are presented as Mean ± SEM, n = 5 independent wells. In conditions with WNT3A CM, $p < 0.05$ for 2.5 μM simva compared to DMSO (labeled as 0 μM simva) and $p < 0.05$ for 5 μM simva compared to DMSO, using Student's unpaired t-test.

Figure S4, related to Figure 4.



The siRNAs used for *HMGCR* knockdown are effective at reducing expression of this gene. SH-SY5Y cells were transfected with control (non-targeting) siRNA or one of three independent siRNAs targeting *HMGCR*. 72 hours later *HMGCR* expression was quantified by RT-PCR analysis. Results are presented as Mean ± SEM, n = 3 independent wells. Using Student's unpaired t-test, $p < 0.05$ for each of the following pairs of siRNAs: Control vs HMGCR_1, Control vs HMGCR_2, and Control vs HMGCR_3.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

aNPC Culture

aNPCs were derived from the DG of 8-10 week old C57BL/6J mice. aNPCs were grown in suspension as neurospheres in proliferation media: DMEM/F-12 + Glutamax (Life), 2% B27-Supplement (Life), 1% Penicillin/Streptomycin (Sigma-Aldrich), 20 ng/ml basic fibroblast growth factor (FGF, Life), 20 ng/ml epidermal growth factor (EGF, PeproTech, Rocky Hill, NJ, www.peprotech.com), 2 mM L-glutamine (Life), 10 mM HEPES Buffer Solution (Life). For differentiation assays, Clear Bottom 96 Well Imaging Plates (Corning) were coated with laminin (Life) for 4 hours followed by Poly-L-Ornithine Solution (Sigma-Aldrich) overnight at 37°C. Cells were dissociated in Accutase (Life) and plated in differentiation media: similar to proliferation media above but without EGF and FGF, and with 5 μM forskolin (Sigma-Aldrich), 1 μM retinoic acid (Sigma-Aldrich), and 20ng/mL rcWNT3A (Millipore).

SH-SY5Y Cell Culture

SH-SY5Y cells were purchased from the American Type Culture Collection, and cultured in DMEM with 10% FBS (Life Technologies).

Transcriptional Reporter

The BAR reporter is a lentiviral vector containing 12 transcription factor binding sites that respond to activation of the WNT (5'-AGATCAAAGG-3'), driving expression of

either Venus or Luciferase as indicated. Reporter cell lines were generated by lentiviral infection and subsequent puromycin selection.

Compounds

The following compounds were used in this study: rcWNT3A (Millipore), simvastatin (Cayman Chemical), XAV939 (Selleck Chemical), 5-ethynyl-2'-deoxyuridine (EdU) (Life), squalene (Sigma-Aldrich), farnesyl pyrophosphate (Sigma-Aldrich), and geranylgeranyl pyrophosphate (Sigma-Aldrich). XAV939 inhibits WNT signaling by promoting stability of Axin1 (Huang et al., 2009).

RT-PCR analysis

RNA was purified using RNeasy Mini Kit (Qiagen) and Trizol (Life) for cell samples and hippocampi, respectively, according to the manufacturers' instructions. Following DNAse treatment, cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Light Cycler FastStart DNA Master SYBR Green I (Roche) was used for RT-PCR, with a Light Cycler 480 instrument. Copy numbers were determined by absolute quantification and normalized to *Gapdh (or GAPDH in human cells)*. Primers used for RT-PCR amplification are listed in the supplemental experimental procedures.

Mouse Tissue Collection

For tissue collection for staining, animals were deeply anesthetized and perfused transcardially with 10 ml of 0.9% saline followed by 50 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and post-fixed in

PFA overnight at 4°C, rinsed in PB, and transferred to 30% sucrose for 48 hours. Brains were mounted on a sliding microtome and cut into 40 μm coronal sections throughout the DG (bregma -1.2 mm to bregma -2.8 mm).

For RNA collection, animals were euthanized by cervical dislocation, and hippocampi were immediately dissected and dissolved in Trizol.

Antibody Staining

Antigen retrieval was performed on tissue samples by heating to 80º C in 10mM sodium citrate buffer for 30 minutes. To label cells that had incorporated EdU we used the Click-iT® EdU Alexa Fluor 647 Imaging Kit (Life) according to the manufacturer's instructions.

Cell plates (aNPC experiments) or tissue sections were washed in PBS, then permeabilized and blocked in 5% goat serum (Life) and 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 1 hour at room temperature. Samples were then incubated with primary antibodies overnight at 4°C. The following day samples were washed with 0.1% Triton X-100 in PBS and then incubated with secondary antibodies for 1 hour at room temperature. Cell plates were then washed and Hoechst 33342 Dye was added in PBS. Tissue sections were washed and then incubated in DAPI (Sigma-Aldrich) in PBS for 15 minutes. Tissue sections were rinsed in PB and mounted on Superfrost microscope slides (VWR) using Mowiol (Sigma-Aldrich).

The following antibodies were used in this study for immunofluorescence experiments: ß-galactosidase 1:400 (Promega), DCX 1:200 (Abcam), TUJ1 1:400 (Promega), GFAP 1:1000 (Dako), MCM2 1:400 (Abcam), Nestin 1:400 (Abcam), NEUN 1:1000 (Millipore), S100B 1:200 (Abcam), Cleaved Caspase 3 1:50 (Cell Signaling), Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) 1:1000 (Life), Alexa Fluor 647 Goat Anti-Rabbit IgG (H+L) at 1:1000 (Life). To label cells that had incorporated EdU we used the Click-iT® EdU Alexa Fluor 647 Imaging Kit (Life) according to the manufacturer's instructions.

Imaging and Quantification

BAR Venus aNPCs were imaged with a fluorescence microscope (Nikon) using a 20x objective. GFAP and TUJ1 stained aNPCs were imaged with a Nikon A1 confocal microscope using a 20x objective. Venus$^+$ or GFAP$^+$ and TUJ1$^+$ cells as well as Hoechst stained nuclei were counted in 5 fields per well from 3 wells per condition using ImageJ. The percent of marker$^+$ cells was determined by dividing by total number of nuclei, and was averaged across wells.

The density of ß-gal$^+$ nuclei was quantified using the optical fractionator method of the Stereo Investigator software package (MBF Bioscience) in conjuction with semi-automating counting using a Zeiss Axioskop 2 plus fluorescence microscope (Carl Zeiss International) set to a 40x objective. A contour was drawn around the DG and we counted the number of ß-gal stained nuclei in 6-7 sections spaced 240 μm apart per mouse. The

density of ß-gal$^+$ nuclei was averaged across all sections per mouse, and then averaged for all mice per treatment group.

To quantify MCM2$^+$, DCX$^+$, EdU$^+$/DCX$^+$, Nestin$^+$/MCM2$^+$, and cCASP3$^+$ cells we collected Z stacks of 6 sections spaced 240 μm apart per mouse with a Nikon A1 confocal microscope using a 20x objective. Single or double positive cells were counted manually using ImageJ. To determine cell number per DG, the sum of the counted cells was multiplied by 6 and then averaged for all mice per treatment group.

Luciferase Reporter Assay

BAR-luciferase reporter cells were analyzed 24 hours after treatment (or at the time specified) using the Dual-Luciferase Reporter Assay System (Promega) and an Envision multi-label plate reader (Perkin Elmer) according to the manufacturers' instructions.

siRNA Transfection

siRNAs were purchased from Qiagen and reverse-transfected at a final concentration of 10 nM using RNAiMAX (Life) according to the manufacturer's instructions. Drugs and CM were added to cells 48 hours post-transfection. Luciferase assays were performed 72 hours post-transfection.

RT-PCR primers

mouse *Gapdh* (AGGTCGGTGTGAACGGATTTG /

TGTAGACCATGTAGTTGAGGTCA); mouse *Axin2*

(TGACTCTCCTTCCAGATCCCA / TGCCCACACTAGGCTGACA); mouse *CyclinD1*

(GCATGTTCGTGGCCTCTAAG / GTAGATGCACAACTTCTCGGC); *LacZ*

(CGCTGACGGAAGCAAAACA / GCCCGGATAAACGGAACTG); mouse *Tuj1*

(TATGAAGATGATGACGAGGAATCG / TACAGAGGTGGCTAAAATGGGG);

mouse *NeuroD1* (TTAAATTAAGGCGCATGAAGGCC /

GGACTGGTAGGAGTAGGGATG); mouse *Gfap* (CCAAGCCAAACACGAAGCTAA

/ CATTTGCCGCTCTAGGGACTC); mouse *Aqp4* (CTTTCTGGAAGGCAGTCTCAG /

CCACACCGAGCAAAACAAAGAT); human *GAPDH*

(GGAGCGAGATCCCTCCAAAAT / GGCTGTTGTCATACTTCTCATGG); human

HMGCR (TGATTGACCTTTCCAGAGCAAG / CTAAAATTGCCATTCCACGAGC)

siRNA sequences

si:HMGCR_1 (CCGAGCCTAATGAAAGGGAAA); si:HMGCR_2

(CAGAATTTACGTCAACTTGGA); si:HMGCR_3

(AGAGGCTATGATTGAGGTCAA)

REFERENCES

Huang, S.-M.A., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., Hild, M., Shi, X., Wilson, C.J., Mickanin, C., Myer, V., Fazal, A., Tomlinson, R., Serluca, F., Shao, W., Cheng, H., Shultz, M., Rau, C., Schirle, M., Schlegl, J., Ghidelli, S., Fawell, S., Lu, C., Curtis, D., Kirschner, M.W., Lengauer, C., Finan, P.M., Tallarico, J.A.,

Bouwmeester, T., Porter, J.A., Bauer, A., Cong, F., 2009. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461, 614–620.